Supplementary Table 1. The influence of bortezomib on Photofrin uptake by tumor cells

	Photofrin uptake [ng/mg of proteins]		
	Controls	**Bortezomib 1**	**Bortezomib 2**
HeLa cells	**534 ± 136**	**397 ± 33**	**228 ± 78**
EMT6 cells	**321 ± 49**	**376 ± 56**	**394 ± 63**
C-26 cells	**279 ± 46**	**225 ± 49**	**257 ± 55**

For the determination of the proteasome inhibitors influence on Photofrin uptake by tumor cells 1 x 10^6 of C-26, HeLa or EMT6 cells were seeded into a 10-cm plates and allowed to attach overnight. The following day Photofrin at 10 μg/ml concentration and bortezomib at two different concentrations were added to the cell cultures for 24 h incubation in the dark (**1** = 1 ng/ml, 2 ng/ml and 4 ng/ml for C-26, EMT6 and HeLa cells, respectively, and **2** = 2 ng/ml, 4 ng/ml and 8 ng/ml for C-26, EMT6 and HeLa cells, respectively). Next, the cells were harvested, centrifuged at 1300 rpm for 10 min at 4^0C and lysed in 100 μl of RIPA buffer enriched in a Complete® protease inhibitors mixture (Roche). Protein concentration in lysates was estimated using Bradford reagent (Bio-Rad). Lysates were then resuspended in a buffer (1N NaOH and 1% SDS in distilled water) to prevent Photofrin aggregation. Immediately after preparation Photofrin concentrations in samples were measured using Hitachi F-7000 fluorescence spectrophotometer at excitation wavelength of 403 nm and emission wavelength of 627 nm, and compared to standard curves. The standard curves were prepared by adding known amounts of Photofrin to already lysed and not previously

treated tumor cells. Each experimental group was made in independent triplets. The results show the amount of Photofrin (in ng) per mg of proteins in each experimental sample.

Supplementary Materials and Methods

Cytotoxic assays

Cell cultures for *in vitro* experiments were illuminated with either He-Ne laser at 632.8-nm (Amber, Warsaw, Poland) or with a 50 W sodium lamp (Phillips) through a red filter as described (1, 2), or as described in (3) when hypericin was used as the photosensitizer. Briefly, tumor cells were dispensed into a 24-well flat-bottomed plate at a concentration of 5 x 10^3 cells/well and allowed to attach overnight. Then, cells were treated with investigated compounds or with a control medium. After a 24-h incubation with 10 μg/ml Photofrin or indicated photosensitizers, the medium in each well was replaced with PBS, and each well was exposed to laser light. The illumination area matched the size of the wells. After the illumination PBS was removed, cells were trypsinized and seeded into a 96-well microtiter plate. Alternatively, tumor cells were dispensed into 35-mm plates at a concentration of 2.5 × 10^5 cells/dish and allowed to attach overnight, followed by addition of Photofrin or indicated photosensitizers, and illumination with a sodium lamp. For the evaluation of cytotoxic effects crystal violet staining was used as described previously (2, 4).

Western blotting

For Western blotting analysis cells were cultured with 10 μg/ml Photofrin for 24 h before illumination. After washing with PBS, the cells were illuminated with a 50 W sodium lamp using red filter. At indicated times the cells were washed with PBS and lysed with RIPA buffer (50 mM Tris base, 150 mM NaCl, 1% NP40, 0.25% sodium deoxycholate, and 1 mM EDTA) supplemented with Complete® protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentration was

measured using BCA protein assay (Pierce, Rockford, IL). Equal amounts of proteins were separated on 12% SDS-polyacrylamide gel, transferred onto Protran® nitrocellulose membranes (Schleicher and Schuell BioScience Inc., Keene, NH, USA), blocked with TBST [Tris buffered saline (pH 7.4) and 0.05% Tween 20] with 5% nonfat milk and 5% FBS. The following antibodies were used for the overnight incubation: anti-HA.11 (mouse monoclonal, Covance, Princeton, NJ), anti-GFP (mouse monoclonal, Covance), anti-ubiquitin (mouse monoclonal, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-actin (rabbit polyclonal, Sigma), anti-KDEL/BiP (mouse monoclonal, Stressgen, Ann Arbor, MI), anti-α-calnexin (mouse monoclonal, Stressgen). After extensive washing with TBST the membranes were incubated for 45 min in corresponding HRP-coupled secondary antibodies (Jackson Immuno Research, West Grove, PA). The reaction was developed using SuperSignal WestPico Kit® (Pierce). After scanning, densitometric analysis of Western blots was performed using the Image Quant 5.2 software (Amersham Bioscience, Piscataway, NJ).

Protein carbonylation assay

ROS-induced protein lesions include formation of carbonyl groups, which can be detected using 2,4-DNPH, a reagent that specifically binds to protein carbonyl groups, and can be revealed immunochemically using specific antibodies (5). Cells were washed with PBS and suspended in a buffer consisting of 10 mM HEPES, 1.1 mM KH_2PO_4, 137 mM NaCl, 0.6 mM $MgSO_4$, 4.4 mM KCl, 1.1 mM EDTA supplemented with Complete® protease inhibitors. Ten-microgram samples of proteins were precipitated with 10% TCA. The precipitates were treated with either 2N HCl alone (control) or 2N HCl containing 5 mg/ml 2,4-DNPH at RT for 30 min. The

resulting hydrazones were precipitated in 10% TCA and then washed three times with ethanol-ethyl acetate (1:1). Final precipitates were dissolved in 6 M guanidine chloride. Equal amounts of whole-cell proteins were separated on 12% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes blocked with TBST [Tris-buffered saline (pH 7.4) and 0.05% Tween 20] supplemented with 5% nonfat milk. Anti-DNPH antibodies (Sigma) in dilution of 1:20 000 were used for a 2 h incubation. After washing with TBST, the membranes were incubated with horseradish peroxidase-coupled secondary antibodies. The reaction was developed using SuperSignal WestPico Kit® (Pierce).

Immunoprecipitation

For immunoprecipitation, cells collected from 60-mm plates 24 hours after PDT were lysed in 1 ml of lysis buffer (50 mM HEPES–KOH, pH 7.4 at 4°C, 100 mM NaCl, 1.5 $MgCl_2$ and 0.1% NP-40) using repeated freeze-thaw cycles. After centrifugation (15 min, 16 000 × g at 4°C), protein concentration was determined with BCA protein assay (Pierce) and all lysates were diluted to the same concentration. Next, 1 ml of lysate from each group was precleared with agarose beads, incubated with 5 µl of antibodies for 1 h at 4°C on a rotary wheel, and then 50 µl of protein G bead slurry (HiTrap™, Amersham Pharmacia Biotech AB, Uppsala, Sweden) was added for overnight incubation at 4°C on a rotary wheel. Afterwards, beads were washed 5 times in lysis buffer. Antigen-bound antibodies were released from protein G by 5 min wash in 0.1 M glycine (pH 2.6) and the suspension was used for detection of protein carbonylation.

Transmission electron microscopy

For transmission electron microscopy (TEM) cells collected from 100-mm plates were fixed in 3% glutaraldehyde, and postfixed in 1% OsO_4 both in the 0.1 M cacodylate buffer pH 7.4, dehydrated in increasing concentrations (50-100%) of ethanol and in propylene oxide and embedded in Poly/Bed® 812 (Polysciences, Inc., Warrington, PA). Resin blocks were cut with a diamond knife on a RMC type MTXL ultramicrotome. Ultrathin sections were mounted on Formvar carbon-coated grids, stained with lead citrate and uranyl acetate, and observed in a Jeol JEM-100S transmission electron microscope (Jeol, Tokyo, Japan).

RT-PCR

RNA was isolated using Chomczynski's modified method (6) from HeLa or C-26 cells after treatment with PDT and/or proteasome inhibitors. RT-PCR was performed with AMV reverse transcriptase (Promega) according to manufacturers protocol. Next, PCR was performed using GoFlexiTaq DNA Polymerase (Promega, Madison, WI) using pairs of primers amplifying huXBP1: 5'-CCT TGT AGT TGA GAA CCA GG-3' (forward), 5'-GGG GCT TGG TAT ATA TGT GG-3' (reverse), and muXBP1: 5'-CCT TGT GGT TGA GAA CCA GG-3' (forward), 5'-GAG GCT TGG TGT ATA CAT GG-3' (reverse) as described before (7). For the XBP-1 transcript, the primers are complementary to the region that includes the 26-base pair deletion dependent on IRE-1 endonuclease activity (8). Primers used for amplification of human and murine actin were: 5'-TTC CTT CCT GGG CAT GGA GT-3' (forward) and 5'-ATC CAC ATC TGC TGG AAG GT-3' (reverse).

Immunofluorescence microscopy

For immunofluorescence microscopy the cells were dispensed in 8-well chamber slides (Nunc, Roskilde, Denmark) and cultured with 10 μg/ml of Photofrin and other reagents for 24 h before illumination. After washing with PBS, the cells were exposed to laser light. After consecutive 24 h of culture in the fresh medium the cells were washed with PBS. Slides were methanol-fixed for 30 min in -20°C, blocked with 5% normal donkey serum and incubated overnight at 4°C with primary antibodies [anti-α_1AT (Sigma), anti-Sec61α, anti-ubiquitin FK2 (both from Stressgen), anti-HA.11, and anti-GFP (both from Covance)] in 5% normal donkey serum in PBS. Slides were washed three times in PBS and incubated with donkey anti-mouse Alexa555- or donkey anti-rabbit Alexa 488-conjugated antibody (Invitrogen-Molecular Probes, Carlsbad, CA; 1:200 for 2 h at room temperature). The slides were washed, mounted in DAPI-enriched Vectashield (Vector Laboratories, Burlingame, CA) and observed under fluorescence confocal microscope (Leica TCS SP2).

Proteasome activity

The 20 S proteasomes were isolated from T2 cells as described (9). Briefly, cells were lysed with 1 mM dithiothreitol, and the stroma-free supernatant was applied to DEAE-Sepharose (Toyopearls). Then, 20S proteasome was eluted with a NaCl gradient in TEAD (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM azide, and 1 mM dithiothreitol) from 100 to 350 mM NaCl. The 20S proteasome was concentrated by ammonium sulfate precipitation (between 40 and 70% of saturation) and separated in a 10–40% sucrose gradient by centrifugation at 40,000 rpm for 16 h (SW40; L7; Beckman & Coulter, Fullerton, CA). Finally, 20S proteasome was purified on MonoQ column and eluted with a NaCl gradient at 280 mM NaCl. The fractions containing purified 20S proteasome were dialyzed against 50 mM NaCl in TEAD and stored on

ice. The purity was determined by SDS-PAGE. One hundred nanograms of purified proteasomes were incubated with 10 μg/ml Photofrin for 30 min. Then, the mixtures were illuminated with 632.8 nm He-Ne ion laser (Amber, Warsaw, Poland) and proteasome activities were determined with 100 μM fluorogenic substrates: Z-Gly-Gly-Arg-βNA (Z = benzyloxycarbonyl and βNA = β-naphtylamide) for tryptic-like activity, Z-Leu-Leu-Glu-βNA for caspase-like activity, and Suc–Leu-Leu-Val-Tyr-AMC (Suc = succinyl, and AMC = 7-amido-4-methylcoumarin) for chymotryptic-like activity. All peptides were purchased from Bachem (Weil am Rhein, Germany). For the measurements of proteasome activity in total cellular lysates C-26 cells were seeded in 100-mm plates at a density of 2×10^6 cells/plate. After incubation with Photofrin for 24 h cells were washed with PBS and illuminated with the sodium light. Then, at indicated time points cells were washed with PBS and lysed [20 mM Tris-HCl (pH 6.8), 50 mM NaCl, 2 mM $MgCl_2$, 0.1% NP40, and Complete® protease inhibitors coctail (Roche)]. Protein content was estimated by BCA (Pierce) and proteasome activities were determined as above.

Supplementary Figure Legends

Supplementary Figure S1. PDT induces ER stress and unfolded protein response.

[A] HeLa cells were incubated with 10 μg/ml of Photofrin for 24 h and exposed to 1.2 J/cm^2 of light. At indicated time points total cell lysates were prepared from tumor cells, and Western blot analysis was performed using anti-BiP, anti-calnexin or anti-β-actin antibodies. MG132 at 250 nM concentration was used as a positive control.

[B] Densitometric analysis of BiP and calnexin expression in HeLa cells from three independent experiments.

[C] HeLa and C-26 cells were incubated with 10 μg/ml of Photofrin for 24 h and exposed to 2.4 or 3.6 J/cm^2 of light. At indicated time points mRNA was isolated and RT-PCR was perform to detect alternative XBP1 splicing. Tunicamycin at a 10 μM concentration was used as a positive control for 8 h incubation.

Supplementary Figure S2. Electron microscopy of HeLa and EMT6 cells treated with PDT.

EMT6 cells were incubated with 10 μg/ml of Photofrin for 24 h and exposed to 2.4 J/cm^2 of light. For electron microscopy cells were collected and fixed 24 h after PDT as described under Materials and Methods section. Black arrows indicate distended ER, black arrowhead indicates autophagosomal structures, red arrows indicate swollen mitochondria, and red arrowheads show autophagosomes/lysosomes containing mitochondrial debris.

Supplementary Figure S3. Bortezomib increases accumulation of ER-membrane associated proteins in PDT-treated cells.

[A] HeLa cells stably transfected with expression plasmids encoding reporter proteins (α1AT, δCD3 and αTCR) were incubated with 4 ng/ml of bortezomib and/or with 10 μg/ml of Photofrin for 24 h and exposed to 1.2 J/cm^2 of light. Indirect immunofluorescence microscopy was performed using fluorescence microscope 24 h after illumination with anti-α1AT (green), anti-HA-tag (to detect δCD3 and αTCR) primary antibodies (red).

[B] HeLa cells were incubated with 10 μg/ml of Photofrin and/or 4 ng/ml of bortezomib for 24 h and exposed to 1.2 J/cm^2 of light. After a 24 h incubation immunopreciptiation was performed with whole tumor cell lysates using anti-ubiquitin antibodies. Protein carbonylation was determined by DNPH method as described in Materials and Methods.

[C] HeLa cells stably transfected with expression plasmids encoding δCD3 were incubated with 4 ng/ml of bortezomib and/or with 10 μg/ml of Photofrin for 24 h and exposed to 1.2 J/cm^2 of light. After a 24 h incubation immunopreciptiation was performed with whole tumor cell lysates using anti-HA antibodies. Protein carbonylation was determined by DNPH method as described in Materials and Methods. Equal amounts of immunoprecipitates were analysed using Western blotting for detection of δCD3 with anti-HA antibodies.

Supplementary Figure S4. Time-course changes in tumor cells pre-treated with MG132 for either 1 h or 24 h and exposed to PDT.

HeLa cells were incubated for 24 hours with 10 μg/ml Photofrin. MG132 at 250 nM concentration was added together with Photofrin or for the last 1 h of incubation.

Tumor cells were then illuminated with light at a fluence of 1.2 or 2.4 J/cm^2 and photographed under inverted microscope at indicated times after illumination.

Supplementary Figure S5. Accumulation of undegraded proteins and ER stress sensitize tumor cells to PDT-mediated cytotoxicity.

[A] HeLa cells were incubated for 24h with 10 μg/ml Photofrin. MG132 at 250 nM concentration was added together with Photofrin or for the last 1h of incubation. Tumor cells were then illuminated with light at a fluence of 1.2 or 2.4 J/cm^2 and cytotoxic effects were measured with crystal violet staining 8h after illumination. The bars represent percent survival versus untreated controls. Data refer to mean±SD. *p<0.05 versus single modality (PDT or MG132) treated cells (Student's *t*-test).

[B] HeLa cells were incubated for 24h with 10 μg/ml Photofrin. MG132 at 250 nM concentration was added together with Photofrin or for the last 1h of incubation. Tumor cells were then illuminated with light at a fluence of 1.2 J/cm^2 and the level of protein carbonylation was measured as described earlier.

Supplementary Figure S6. Cytotoxic effects of PDT with other photosensitizers combined with proteasome inhibitors.

HeLa cells were incubated for 1 h with 5 μg/ml Verteporfin **[A]**, 6 h with 10 mM ALA **[B]** or 24 h with 50 nM Hypericin **[C]** with or without bortezomib. Tumor cells were then illuminated with light at indicated light fluencies. Cytotoxic effects were measured with crystal violet staining 24 h after illumination. The bars represent percent survival versus untreated controls. Data refer to mean ± SD. *p<0.05 versus single modality (PDT or bortezomib) treated cells (Student's *t*-test).

Supplementary References

1. Golab J, Nowis D, Skrzycki M*, et al.* Antitumor effects of photodynamic therapy are potentiated by 2-methoxyestradiol. A superoxide dismutase inhibitor. J Biol Chem 2003;278:407-14.
2. Nowis D, Legat M, Grzela T*, et al.* Heme oxygenase-1 protects tumor cells against photodynamic therapy-mediated cytotoxicity. Oncogene 2006;25:3365-74.
3. Hendrickx N, Volanti C, Moens U*, et al.* Up-regulation of cyclooxygenase-2 and apoptosis resistance by p38 MAPK in hypericin-mediated photodynamic therapy of human cancer cells. J Biol Chem 2003;278:52231-9.
4. Makowski M, Grzela T, Niderla J*, et al.* Inhibition of cyclooxygenase-2 indirectly potentiates antitumor effects of photodynamic therapy in mice. Clin Cancer Res 2003;9:5417-22.
5. Nakamura A, Goto S. Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis. J Biochem 1996;119:768-74.
6. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.
7. Nowis D, McConnell E, Wojcik C. Destabilization of the VCP-Ufd1-Npl4 complex is associated with decreased levels of ERAD substrates. Exp Cell Res 2006;312:2921-32.
8. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell 2001;107:881-91.

9. Mlynarczuk-Bialy I, Roeckmann H, Kuckelkorn U*, et al.* Combined effect of proteasome and calpain inhibition on cisplatin-resistant human melanoma cells. Cancer Res 2006;66:7598-605.